Synthesis of a polymyxin derivative for photolabeling studies in the gram-negative bacterium Escherichia coli

Robinson, J A ; van der Meijden, B

Abstract: The antimicrobial activity of polymyxins against Gram-negative bacteria has been known for several decades, but the mechanism of action leading to cell death has not been fully explored. A key step after binding of the antibiotic to lipopolysaccharide (LPS) exposed at the cell surface is 'self-promoted uptake' across the outer membrane (OM), in which the antibiotic traverses the asymmetric LPS-phospholipid bilayer before reaching the periplasm and finally targeting and disrupting the bacterial phospholipid inner membrane. The work described here was prompted by the hypothesis that polymyxins might interact with proteins in the OM, as part of their self-promoted uptake and permeabilizing effects. One way to test this is through photolabeling experiments. We describe the design and synthesis of a photoprobe based upon polymyxin B, containing photoleucine and an N-acyl group with a terminal alkyne suitable for coupling to a biotin tag using click chemistry. The resulting photoprobe retains potent antimicrobial activity, and in initial photolabeling experiments with Escherichia coli ATCC25922 is shown to photolabel several OM proteins. This photoprobe might be a valuable tool in more detailed studies on the mechanism of action of this family of antibiotics.

DOI: https://doi.org/10.1002/psc.2736

Synthesis of a Polymyxin Derivative for Photolabeling Studies in the Gram-negative Bacterium *Escherichia coli***

Benjamin van der Meijden and John A. Robinson*

Department of Chemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Running Title: A Polymyxin Derivative for Photolabeling Studies in Bacteria

Correspondence to:

Professor John A. Robinson, Department of Chemistry, University of Zurich,

Winterthurerstrasse 190, 8057 Zurich, Switzerland

Tel: +41 44 635 4242

E-mail: robinson@oci.uzh.ch

**) Invited Article for the Anniversary Issue 2015 of Journal of Peptide Science

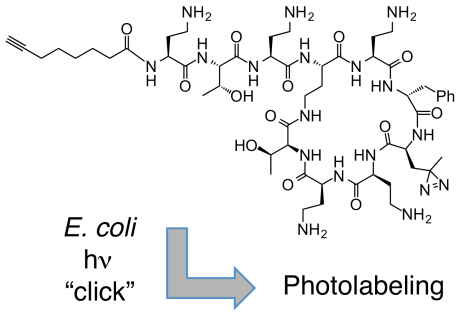
Abstract

The antimicrobial activity of polymyxins against Gram-negative bacteria has been known for several decades, but the mechanism of action leading to cell death has not been fully explored. A key step after binding of the antibiotic to lipopolysaccharide (LPS) exposed at the cell surface is "self-promoted uptake" across the outer membrane (OM), in which the antibiotic traverses the asymmetric LPS-phospholipid bilayer before reaching the periplasm and finally targeting and disrupting the bacterial phospholipid inner membrane. The work described here was prompted by the hypothesis that polymyxins might interact with proteins in the OM, as part of their self-promoted uptake and permeabilizing effects. One way to test this is through photolabeling experiments. We describe the design and synthesis of a photoprobe based upon polymyxin B, containing photoleucine and an N-acyl group with a terminal alkyne suitable for coupling to a biotin tag using click chemistry. The resulting photoprobe retains potent antimicrobial activity, and in initial photolabeling experiments with *Escherichia coli* ATCC25922 is shown to photolabel several OM proteins. This photoprobe might be a valuable tool in more detailed studies on the mechanism of action of this family of antibiotics.

Key words: peptide, antibiotic, membrane protein, polymyxin, mechanism of action, click reaction

Graphical Table of Contents

A combined solid-phase peptide synthesis and solution macrocyclization approach to a photolabeling probe based upon polymyxin B is described. The probe contains a diazirine (photoleucine) and an alkyne for conjugation using click chemistry. The probe photolabels outer membrane proteins in *Escherichia coli*.



Introduction

The polymyxins comprise a small family of macrocyclic cationic/hydrophobic decapeptide antibiotics with an N-terminal fatty acid chain [1] (Figure 1), which are produced naturally by *Bacillus sp*. [2,3]. They are used clinically for the treatment of serious infections caused by multi-drug resistant Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and *Klebsiella* sp., despite earlier concerns about nephrotoxicity and neurotoxicity [4,5]. The mechanism of action of polymyxins has been extensively studied, but still remains incompletely understood. The selective antimicrobial activity against Gram-negative bacteria arises due to an initial interaction of the cationic polymyxins with the negatively charged lipid A portion of lipopolysaccharide (LPS) in the outer leaflet of the asymmetric outer membrane (OM). NMR studies have provided models for the polymyxin-LPS complex [6], with the binding site lying within the negatively charged lipid A head-group of LPS (reviewed in [3]). Passage across the OM is proposed to occur by self-promoted uptake, in which the polymyxins exploit their own potent permeabilizing effect on the LPS-phospholipid bilayer to traverse the OM [7-9]. After gaining access to the periplasm, the antibiotic can bind and disrupt the inner cytoplasmic membrane (CM) in a process, which may include membrane depolarization, mixing of lipid components between bilayers, and leakage of contents from the cytoplasm, and culminates in cell death [10-13].

Nevertheless, the relationships between bacterial killing and the interactions of polymyxins with the OM and CM are complex and incompletely understood [14]. Some evidence suggests that the concentrations of polymyxin that result in rapid bacterial killing are insufficient to cause depolarization of the CM [15]. Also, pore formation and depolarization of the CM seem not to be obligatory for the bactericidal action of polymyxin [13]. Moreover, the OM permeabilizing effect of the de-N-acylated form of polymyxin B (called polymyxin

nonapeptide) was shown to be strongly enantioselective, whereas binding to LPS was not [16]. This suggests that another key chiral bacterial receptor-polymyxin interaction in bacteria likely exists. One possibility is that polymyxins might interact with OM proteins in Gram-negative bacteria as part of the mechanism of self-promoted uptake, although this has not been reported to date. In this work, we set out to design and synthesize a photoaffinity probe that might be used to detect interactions between polymyxins and OM proteins in photolabeling experiments.

We report here the design and synthesis of a photoprobe based upon polymyxin B3, and results of initial photolabeling experiments, which indeed reveal its interaction with several OM proteins in *E. coli* ATCC25922.

Materials and Methods

Synthesis. For the synthesis of photoprobe Pal-PMB solid-phase Fmoc chemistry [17] was used with an orthogonal Alloc protecting group on the Dab^4 side chain. Fmoc-Thr(tBu)-OH (0.1 mmol) was first coupled to chlorotrityl chloride resin and the peptide chain was assembled using HBTU/HOBt and $i Pr_2EtN$ in DMF for coupling reactions and piperidine in DMF (20%) for Fmoc-deprotection. L-PhotoLeu-OH was from *Thermo Fisher Scientific* (Rockford, Illinois). Unreacted amines were capped after each coupling step with acetic anhydride, $i Pr_2EtN$ and HOBt in DMF. To remove the Alloc group, the resin was treated with $Pd(PPh_3)_4$ (1 eq.), Bu_3SnH (12 eq.), and *p*-nitrophenol (2 eq.) in dry DCM under nitrogen in the dark for 1 h. The resin was then drained and the process repeated. The resin was washed with DCM (2x), with 0.5% sodium dithiodiethylcarbamate in DMF (2x), dry DMF (3x) and again with DCM (2x). The peptide was then cleaved from the resin with 0.8% TFA in DCM and the acid was immediately neutralized with $i Pr_2EtN$. The solvent was removed, DMF (0.5

ml) was added, and the peptide crude was precipitated with water and collected by centrifugation. The precipitate was taken up in a water-acetonitrile mixture and lyophilized. Cyclization was performed with diphenylphosphoryl azide (Dppa) (0.5 mmol, 5 eq.) and N-methylmorpholine (NMM) (1 mmol, 10 eq.) in DMF and DMSO (2:1) under argon. The crude peptide was then taken up in 2% DBU and 2% piperidine in DMF (3 ml) and stirred for 20 min at room temperature. Ice-cold water was added to precipitate crude product, which was collected by centrifugation and redissolved in MeCN/water and lyophilized. 7-Octynoic acid (3 eq.) was coupled in solution to the peptide using HBTU (2.9 eq.), HOBt (2.9 eq.) and *i*Pr_2EtN (11 eq.) in DMF. The solvent was then removed in vacuo. The crude peptide was then dissolved in TFA/TIS/H_2O (95:2.5:2.5, 5 ml) at 0°C for 40 min. Addition of ice-cold diisopropyl ether precipitated the peptide, which was collected and washed twice with diisopropyl ether. The product was purified by reversed phase HPLC on a Waters XBridge™ (C8, 100 x 19 mm, 5 µm, OBD™) column with a gradient of 10-35% MeCN/H_2O + 0.1% TFA in 4 column volumes. Yield 8.1 mg (7%), >95% purity. Retention time t_R = 8.3 min (*GRACE* VYDAC® 214TP54, C4, 5 µm, 4.6 x 250 mm, flow 1 ml/min, gradient 10-50% MeCN/H_2O + 0.1% TFA over 12.5 min); HR-ESI-MS: m/z ($M+2H^+$) 599.3456 (calc. mass = 599.3467). The ^{1}H NMR spectrum and assignments are in the Supporting Information.

For the synthesis of polymyxin B3 a similar method was used, except that n-octanoic acid was coupled to the peptide-N-terminus on the resin, while macrocyclization was again performed in solution. The product was purified by preparative reverse phase HPLC on a Waters XBridge™ (C8, 100 x 19 mm, 5 µm, OBD™) column with a gradient of 10-35% MeCN/H_2O + 0.1% TFA in 4 column volumes. The product was of >95% purity by reversed phase HPLC. Yield = 5.0 mg (4%). Retention time t_R = 9.9 min (*GRACE* VYDAC® 214TP54, C4, 5 µm, 4.6 x 250 mm, flow 1 ml/min, gradient 10-50% MeCN/H_2O + 0.1% TFA over 12.5

min); HR-ESI-MS: m/z ($M+2H^+$) 595.3744 (calc. mass = 595.3744). The ^1H NMR spectrum and assignments are in the Supporting Information.

Determination of MIC. Antimicrobial activity was assayed against *Escherichia coli* ATCC25922 and *P. aeruginosa* PAO1 using the Clinical and Laboratory Standards Institute broth microdilution method in cation-adjusted Mueller-Hinton-II broth supplemented with 0.02% BSA (*Roche-Applied-Science*, Fraction V, Rotkreuz) [18]. The MIC values are typically 4-8 fold lower when the assays are performed in Mueller-Hinton-I broth in the presence of 0.02% BSA.

Photolabeling experiments. *E. coli* ATCC25922 was grown in Mueller-Hinton II medium at 37 °C to an OD_{600} of 1.0. Cells were resuspended in cold phosphate-buffered saline (PBS) (140 mM NaCl, 10 mM phosphate, pH 7.4). Aliquots of the cell suspension were transferred to borosilicate-flasks, diluted with PBS to a final OD of 1, and incubated with Pal-PMB (1 μg/ml) for 15 min at 37 °C in the dark, and then irradiated under gentle stirring at 37 °C for 15 min in a Rayonet-reactor (350 nm, 16 x 8 W *Sylvania* blacklight 350 F8W/T5/BL350 lamps, in a 10 cm diameter array). After photolysis, the labelled cells were centrifuged (20 min, 4000 rpm), washed once with ice-cold PBS, and the cell pellet could be stored at -20 °C.

Membrane protein isolation was performed by a modified carbonate extraction method [19]. Labelled cells resuspended in phosphate buffer (20 ml, 50 mM, pH 7.4) with benzamidine (1.25 μM), phenylmethanesulfonylfluoride (1 μM) and DTT (1 μM) were disrupted by sonication and unbroken cells removed by centrifugation at 4000 rpm for 20 min. The supernatant was diluted with ice-cold 0.1 M sodium carbonate (30 ml) and incubated with shaking at 100 rpm for 1 h at 4 °C. The membrane precipitate was collected by ultracentrifugation in a *Sorvall* T-865 rotor (*ThermoFisher Scientific*, Rockford, Illinois) at 45000 rpm for 1 h at 4 °C. The supernatant was discarded and the pellet resuspended in

phosphate buffer (50 mM, pH 7.4) and centrifuged again at 45000 rpm for 1 h at 4 °C. This washing step was repeated. The pellet (ca. 30 μl paste) was resuspended in phosphate buffer (100 μl, 50 mM, pH 7.4). Aliquots (29 μl) were taken and phosphate buffer (5μl, 50 mM, pH 7.4) with 14% SDS was added and the sample was heated to 100 °C for 3 min to denature OM proteins. Biotin-PEG_2-N_3 (2.2 μl of 0.15 mM in DMSO), aminoguanidine (5 μl of 5 mM in H_2O) were added and the sample vortexed. $CuSO_4$ (5 μl of 1 mM in H_2O) and 2-[4-{(bis[(1-tert-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino)methyl}-1*H*-1,2,3-triazol-1-yl]acetic acid (BTTAA) (2.5 μl, 2.5 mM in DMSO:H_2O, 1:3) were premixed and then added to the reaction. Ascorbate (5 μl, 5 mM in H_2O) was added and the reaction was shaken at 1000 rpm and 25 °C for 1 h. Aliquots were analyzed after heating and reduction with DTT by SDS-PAGE (8 or 12% cross-linked gels).

Electrophoretic transfer onto a PVDF-membrane (*Millipore* Immobilon-P, 0.45 µm, *Merck*, Darmstadt) was performed using a 1:1 mixture of Tris-glycine-SDS buffer (12 mM Tris, 96 mM glycine, 0.1% SDS) and phosphate-SDS-urea buffer (10 mM Na_2HPO_4, 1% SDS, 6 M urea) (const. I = 1.0 A, max. U = 25 V, 19 min). The membrane was rinsed with water and methanol after transfer and blocked at 4 °C overnight with 3% BSA (Bovine Serum Albumin Fraction V), 0.2% Tween 20 in PBS (140 mM NaCl, 10 mM phosphate, pH 7.4). For biotin detection, the blocked membrane was incubated for 1 h at room temperature with streptavidin-horse radish peroxidase conjugate (*ThermoFisher Scientific*, Rockford, Illinois), diluted 1:30'000 from a stock solution (1 mg/ml) with 3% BSA + 0.2% Tween 20 in PBS. Subsequently, the membrane was washed 4 x 5 min with PBS and incubated for 5 min with chemiluminescence-substrate (*Pierce* Super Signal West Pico, 0.07 ml/cm^2 membrane). Chemiluminescence was detected by exposing the membrane to *Kodak* X-Omat LS film (*VWR*, *Radnor,* PA).

Results and Discussion

Polymyxin B is produced naturally as a mixture containing different fatty N-acyl groups (Figure 1) [1]. Moreover, extensive structure-activity relationship (SAR) studies on synthetic polymyxin B derivatives were available to guide here the design of a photoprobe [3]. For example, the D-Phe^6-L-Leu^7 segment of the macrocycle (Figure 1) forms a hydrophobic and characteristic ß-turn-like structure, and conservative amino acid substitutions at these positions can be made without a major loss of antimicrobial activity (colistin contains D-Leu^6-L-Leu^7) [20]. The L-Leu^7 residue was replaced here by L-photoleucine [21] in the photoprobe Pal-PMB (Figure-1), so as to include a diazirine as a photo-reactive group [22]. Upon irradiation by UV light at ca. 350 nm, the diazirine is converted into a highly reactive carbene suitable for protein labeling [23]. For detection of photolabeled proteins, an alkyne was introduced at the N-terminus in the form of an oct-7-ynoyl group (Figure 1), since fatty acyl chains with 7-9 carbon atoms typically show optimal antimicrobial activity against *E. coli* [3]. After photolabeling, this allows use of "click chemistry" (alkyne + azide) to introduce a biotin derivative [24]. Biotinylated proteins can then be detected after gel electrophoresis by chemiluminescence detection with streptavidin-horse radish peroxidase conjugates. The target of our first synthetic efforts was, therefore, the photoprobe Pal-PMB (Figure 1).

The peptide backbone of Pal-PMB was assembled on 2-chlorotrityl chloride resin using standard Fmoc-chemistry, except that an orthogonal Alloc protecting group was used for the side chain of Dab^4, to allow selective deprotection and subsequent macrocyclization (Scheme 1). However, the N-terminal octynoyl group was not added before macrocyclization, because the alkyne group proved to be sensitive to reduction under conditions typically used for Pd-catalyzed removal of the Alloc group. Instead, an Fmoc group was retained at the N-

terminus of the linear resin-bound decapeptide. The Dab(Alloc)^4 group could then be removed selectively on-resin, and the linear protected peptide was released from the resin with 0.8% TFA in DCM. Subsequently, cyclization was performed in dilute DMF/DMSO solution using diphenylphosphoryl azide for activation and N-methylmorpholine as a base. These conditions suppressed epimerization of the Thr^{10} Cα position, which was a problem when HBTU/HOBT/*i*Pr$_2$NEt was used for cyclization. The Fmoc group was then removed, the octynoyl group was coupled to the N-terminus, and the side chain protecting groups were finally removed using TFA, all in good yield. The photoprobe Pal-PMB was obtained in >98% purity and high-resolution electrospray MS confirmed the expected mass. For later comparisons, polymyxin B3 was also synthesized by analogous methods. The ^{1}H NMR spectrum of Pal-PMB showed for all residues (except PhotoLeu^7), very similar chemical shifts to those of polymyxin B3 in H_2O/D_2O (9:1), pH 3.0 (see supporting information), suggesting that both macrocyclic peptides have similar average backbone conformations in solution. The antimicrobial activity of Pal-PMB and the synthetic polymyxin B3 was measured against *E. coli* ATCC25922 and *P. aeruginosa* PAO1 in Mueller-Hinton II broth in the presence of 0.02% BSA using a standard antimicrobial susceptibility assay [18]. The minimal inhibitory concentration (MIC) for Pal-PMB was 0.12 μg/ml against both *E. coli* and *P. aeruginosa*, whereas for polymyxin B3 the MICs were both 0.06 μg/ml. These results show that the photoprobe retains potent antimicrobial activity against both Gram-negative bacteria.

As an initial proof-of-principle study, a protocol was established to determine by photolabeling whether the probe Pal-PMB interacts with any OM proteins in the test strain *E. coli* ATCC25922. This strain is a clinical isolate that is often used in antimicrobial susceptibility assays, and so was used here for a photolabeling trial. The labeling protocol (Figure 2) included UV-irradiation (350 nm) of cells in the presence of Pal-PMB. A modified carbonate extraction method was then used to isolate OM proteins [25]. A click reaction was

performed with the OM protein fraction dissolved in phosphate buffer with SDS, using the Cu(I)-stabilizing ligand BTTAA [26], the biotin-PEG_2-N_3 derivative shown in Figure 2, $CuSO_4$ and ascorbate as reducing agent. The mixture of membrane proteins was then separated in 8% and 12% cross-linked SDS-polyacrylamide gels, and then transferred by electroblotting onto a PVDF membrane using buffer conditions optimized for large bacterial membrane proteins [27,28]. Finally, biotin-labeled proteins could be detected on the PVDF membrane using a sensitive chemiluminescence assay with horse-radish-peroxidase linked to streptavidin.

The results of the photolabeling experiment, shown in Figure 3, clearly reveal photolabeling of several membrane proteins in the size range 15-40 kDa, ≈50 kDa and ≈100 kDa. A negative control experiment, performed in the same way but without addition of the Pal-PMB photoprobe, showed as expected no chemiluminescence signals. The Coomassie stained gels, also shown in Figure 3, reveal bands in the 15-45 kDa size range for some of the most abundant OM proteins in *E. coli*, including OmpA/C/F. Other less abundant OM proteins in *E. coli* of ≈50 kDa include the maltose and fatty acid transporters LamB and FadL amongst others. OM proteins in the ≈100 kDa size range include a number of OM transporters and OM protein folding catalysts required for the biogenesis of the OM, which are present in cells only at low copy numbers. The relative intensities of the bands seen in these Western blots are unlikely to correspond directly to the relative amounts of individual photolabeled OM proteins, because the intensities can be influenced by the efficiency of transfer from the gel to the membrane during electroblotting, which is influenced by protein size and properties (hydrophobicity). However, the results shown in Figure 3 clearly reveal selective photolabeling of a group of membrane proteins extracted from this *E. coli* strain. A more detailed genomic (the genome sequence of the strain used has so far not been reported) and proteomic study now seems warranted to identify these photolabeled proteins and to

determine whether their interaction with polymyxin might play a role in uptake of the antibiotic and its permeabilizing effect on the OM.

Acknowledgements

The authors thank the Swiss National Science Foundation for financial support and Annelies Meier for technical support.

References

1 Orwa JA, Govaerts C, Busson R, Roets E, Van Schepdael A, Hoogmartens J. Isolation and structural characterization of polymyxin B components. *J. Chromatog. A* 2001; **912**: 369-373.
2 Storm DR, Rosenthal KS, Swanson PE. Polymyxin and Related Peptide Antibiotics. *Annu. Rev. Biochem*. 1977; **46**: 723-763.
3 Velkov T, Thompson PE, Nation RL, Li J. Structure-Activity relationships of polymyxin antibiotics. *J. Med. Chem*. 2010; **53**: 1898-1916.
4 Vaara M. New approaches in peptide antibiotics. *Curr. Opin. Pharmacol*. 2009; **9**: 571-576.
5 Evans ME, Feola DJ, Rapp RP. Polymyxin B sulfate and colistin: Old antibiotics for emerging multiresistant Gram-negative bacteria. *Ann. Pharmacother*. 1999; **33**: 960-967.
6 Mares J, Kumaran S, Gobbo M, Zerbe O. Interactions of Lipopolysaccharide and Polymyxin Studied by NMR Spectroscopy. *J. Biol. Chem*. 2009; **284**: 11498-11506.
7 Vaara M. Agents that increase the permeability of the outer membrane. *Microbiol. Rev*. 1992; **56**: 395-411.
8 Hancock REW, Chapple DS. Peptide Antibiotics. *Antimicrob. Agents and Chemother*. 1999; **43**: 1317-1323.
9 Schroeder G, Brandenburg K, Seydel U. Polymyxin B induces transient permeability fluctuations in asymmetric planar lipopolysaccharide/phospholipid bilayers. *Biochemistry* 1992; **31**: 631-638.
10 Oh JT, Van Dyk TK, Cajal Y, Dhurjati PS, Sasser M, Jain MK. Osmotic stress in viable Escherichia coli as the basis for the antibiotic response by polymyxin B. *Biochem. Biophys. Res. Comm*. 1998; **246**: 619-623.
11 Liechty A, Chen J, Jain MK. Origin of bacterial stasis by polymyxin B in Escherchia coli. *Biochim. Biophys. Acta* 2000; **1463**: 55-64.
12 Clausell A, Garcia-Subirats M, Pujol M, Busquets MA, Rabanal F, Cajal Y. Gram-Negative Outer and Inner Membrane Models: Insertion of Cyclic Cationic Lipopeptides. *J. Phys. Chem. B* 2006; **111**: 551-563.

13 Daugelavičius R, Bakiene˙ E, Bamford DH. Stages of Polymyxin B Interaction with theEscherichia coli Cell Envelope. *Antimicrob. Agents Chemother*. 2000; **44**: 2969-2978.
14 Velkov T, Deris ZZ, Huang JX, Azad MAK, Butler M, Sivanesan S, Kaminskas LM, Dong YD, Boyd B, Baker MA, Cooper MA, Nation RL, Li J. Surface changes and polymyxin interactions with a resistant strain of Klebsiella pneumoniae. *Innate Immunity* 2014; **20**: 350-363.
15 Zhang L, Dhillon P, Yan H, Farmer S, Hancock REW. Interactions of Bacterial Cationic Peptide Antibiotics with Outer and Cytoplasmic Membranes ofPseudomonas aeruginosa. *Antimicrob. Agents Chemother*. 2000; **44**: 3317-3321.
16 Tsubery H, Ofek I, Cohen S, Fridkin M. The Functional Association of Polymyxin B with Bacterial Lipopolysaccharide Is Stereospecific: Studies on Polymyxin B Nonapeptide. *Biochemistry* 2000; **39**: 11837-11844.
17 Atherton E, Sheppard RC *Solid phase peptide synthesis a practical approach*; Oxford University Press: Oxford, UK, 1989.
18 Andrews JM. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother*. 2001; **48 suppl. 1**: 5-16.
19 Nouwens AS, Cordwell SJ, Larsen MR, Molloy MP, Gillings M, Willcox MDP, Walsh BJ. Complementing genomics with proteomics: The membrane subproteome of Pseudomonas aeruginosa PAO1. *Electrophoresis* 2000; **21**: 3797-3809.
20 Kanazawa K, Sato Y, Ohki K, Okimura K, Uchida Y, Shindo M, Sakura N. Contribution of Each Amino Acid Residue in Polymyxin B3 to Antimicrobial and Lipopolysaccharide Binding Activity. *Chem. Pharm. Bull*. 2009; **57**: 240-244.
21 MacKinnon AL, Garrison JL, Hegde RS, Taunton J. Photo-Leucine Incorporation Reveals the Target of a Cyclodepsipeptide Inhibitor of Cotranslational Translocation. *J. Am. Chem. Soc*. 2007; **129**: 14560-14561.
22 Das J. Aliphatic Diazirines as Photoaffinity Probes for Proteins: Recent Developments. *Chem. Revs*. 2011; **111**: 4405-4417.
23 Dubinsky L, Krom BP, Meijler MM. Diazirine based photoaffinity labeling. *Bioorg. Med. Chem*. 2012; **20**: 554-570.
24 Tang W, Becker ML. "Click" reactions: a versatile toolbox for the synthesis of peptide-conjugates. *Chem. Soc. Revs*. 2014; **43**: 7013-7039.
25 Molloy MP, Herbert BR, Slade MB, Rabilloud T, Nouwens AS, Williams KL, Gooley AA. Proteomic analysis of the Escherichia coli outer membrane. *Eur. J. Biochem*. 2000; **267**: 2871-2881.
26 Besanceney-Webler C, Jiang H, Zheng T, Feng L, Soriano del Amo D, Wang W, Klivansky LM, Marlow FL, Liu Y, Wu P. Increasing the Efficacy of Bioorthogonal Click Reactions for Bioconjugation: A Comparative Study. *Angew. Chem. Int. Ed*. 2011; **50**: 8051-8056.
27 Rabilloud T, Adessi C, Giraudel A, Lunardi J. Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1997; **18**: 307-316.
28 Abeyrathne PD, Lam JS. Conditions that allow for effective transfer of membrane proteins onto nitrocellulose membrane in Western blots. *Can. J. Microbiol*. 2007; **53**: 526-532.

Supporting Information

Additional supporting information (NMR spectra) may be found in the online version of this article at the publisher's web site.

Figure and Scheme legends

Figure 1. Structures of polymyxins B1-B3 and the photoprobe Pal-PMB. In Pal-PMB the L-Leu[7] residue is replaced by L-photoleucine (PhotoLeu[7]), and an oct-7-ynoyl fatty acid is added at the N-terminus.

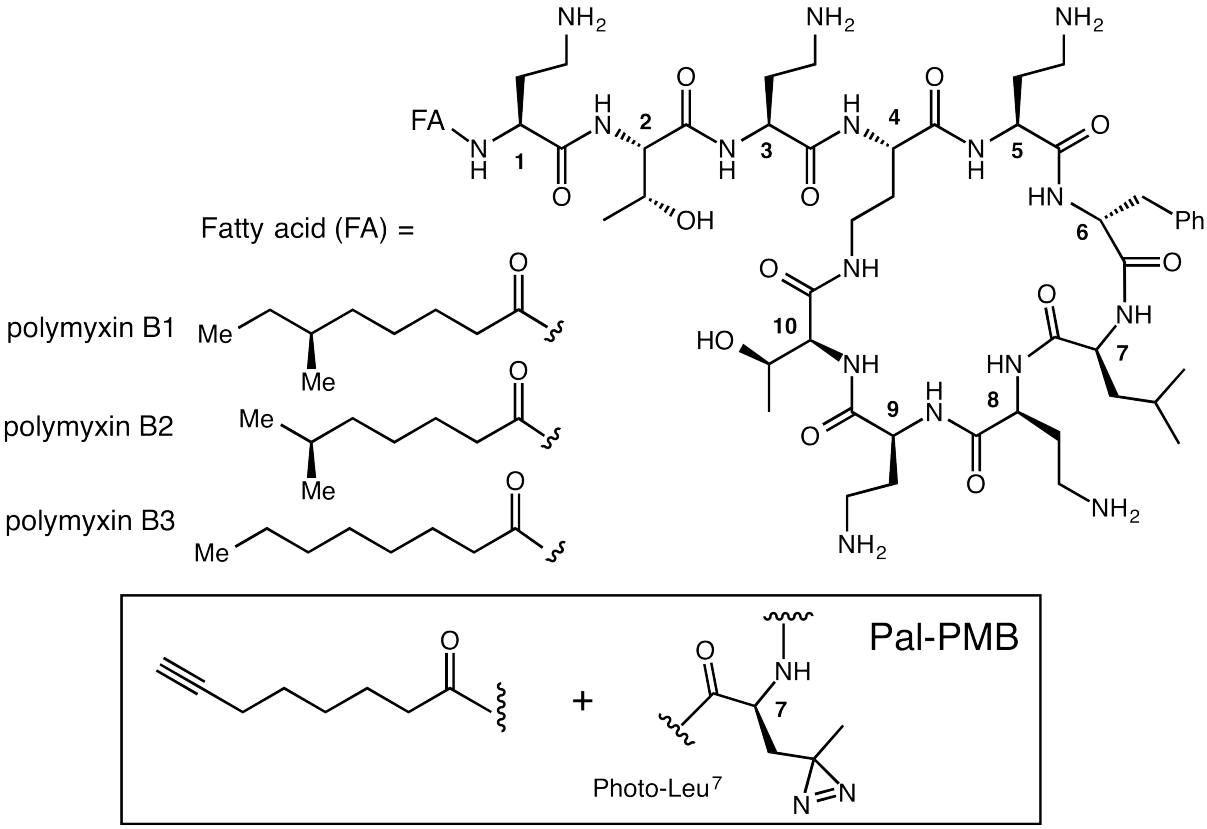


Figure 2. Outline of the photolabeling experiment (see text).

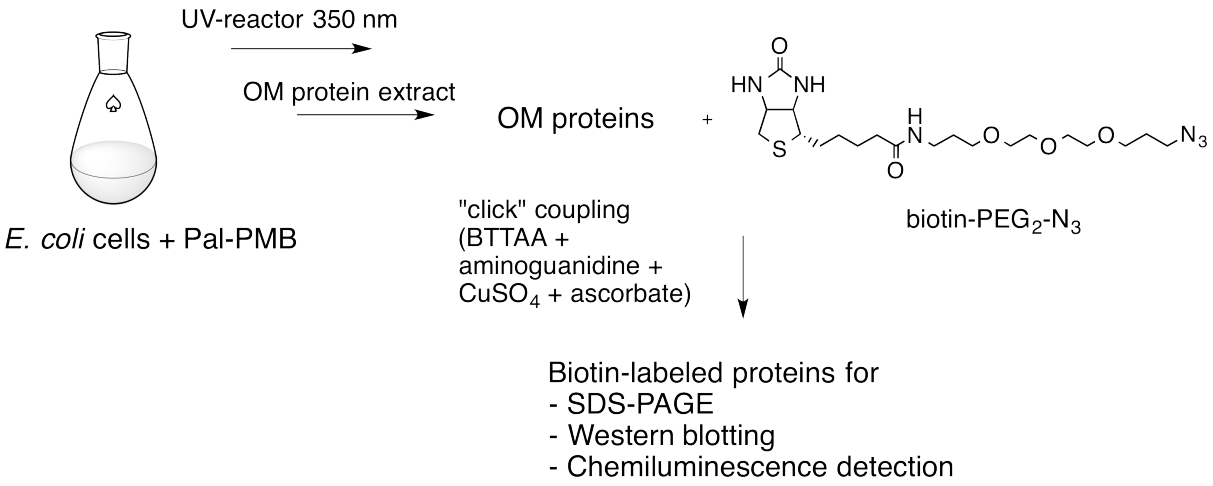
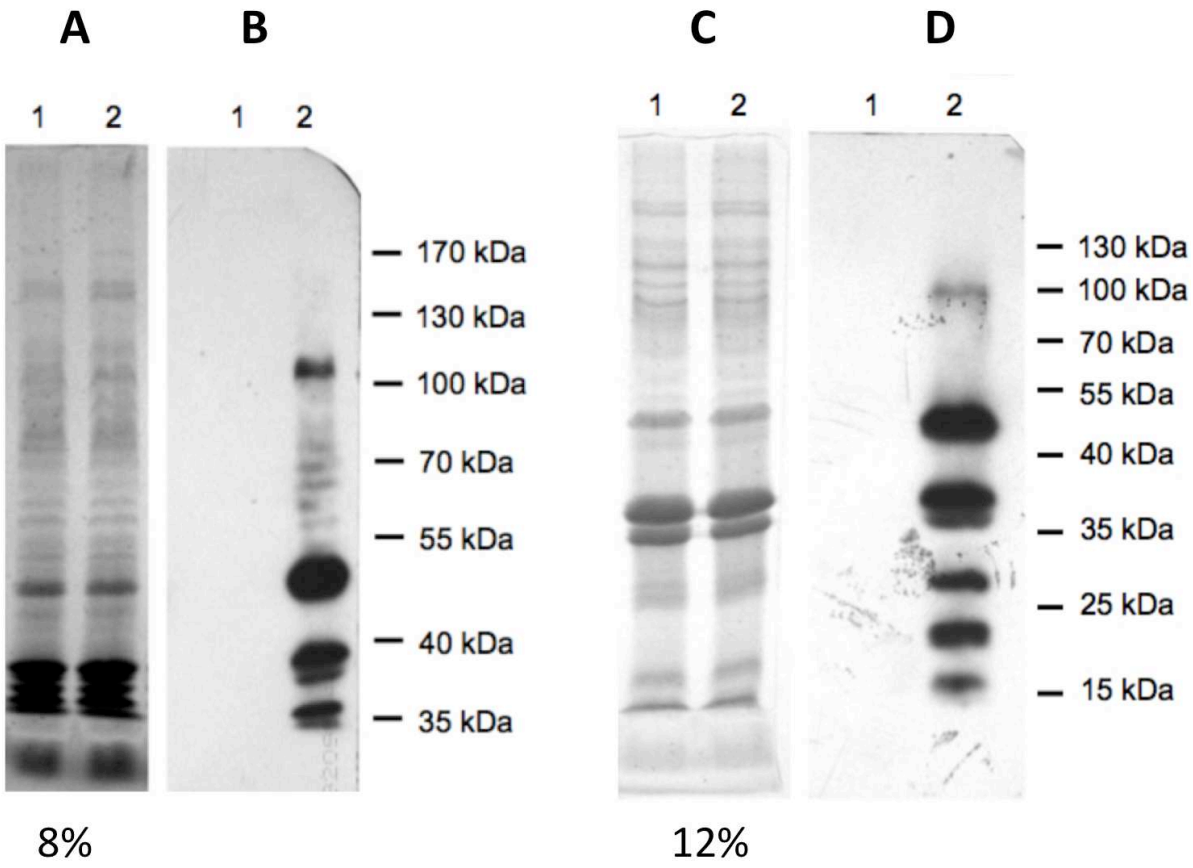


Figure 3. Chemiluminescence detection of biotin-labeled OM proteins after photolabeling (lanes marked 2) compared to a control experiment showing OM proteins isolated without photolabeling (lanes marked 1). **A, C**, show Coomassie stained SDS-PAGE gels (*left* 8% cross-linking, *right* 12% cross-linking); **B, D**, show chemiluminescence detection of biotinylated photolabeled OM proteins after blotting to a PVDF membrane. The 12% gel allows better separation of smaller (<70 kDa) proteins.



Scheme 1. Synthetic route to the photoprobe Pal-PMB.